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Decipher The Role of The Epigenetic Regulators in Progression of Women with Endometriosis - Associated Infertility

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Abstract: **There is continuous increase of inflammatory disease conditions in individuals and it also include a disease called endometriosis. In endometriosis disease, endometrial tissues are present outside the intra-uterine locations having inflammatory properties. HOX genes, are developmental genes, code for proteins and work as critical key regulatory factor during embryogenesis, while epigenetic events regulate gene transcriptions, without changing the underlying DNA sequences, and that includes DNA methyltransferases (DNMTs). Here in this experimental study, the differential expression of HOXA1, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, HOXB6, MLL2, DNMT1, DNMT3A, DNMT3B, PRDM16, G9A and VENTX genes were done. The comparative expression studies of these genes with control, will decipher the role of these genes in diseases progression and further correlating these genes together the molecular pathways can be sketched out.**

Index Terms: **Endometriosis, Infertility, Pelvic Pain, Dysmenorrhea, Dyspareunia**

I. INTRODUCTION

Endometriosis is a chronic inflammatory disease condition in women, where tissues resembling endometrium, usually stromal or glandular, are located outside the uterine cavity (Zondervan et al., 2018). Menorrhagia, dysmenorrhea, dyspareunia, dyschezia, dysuria, pelvic pain, and infertility are the prominent symptoms seen in endometriosis suffering women (Riazi et al., 2015a). In addition, factors like environmental and dietary elements, Genetic and epigenetic immune system, viz. cytokines, interleukins, and intrinsic anomalies in the endometrium, are also associated with the disease (Ashish et al. 2021). Many previous studies have assessed the risk factors associated with endometriosis (Ashish et al., 2021). Age, race, alcohol usage, body mass index, cigarette smoking, and menstrual characteristics such as early age menarche, menstrual length, cycle regularity, dysmenorrhea, and menstrual flow intensity are all associated with the incidences of endometriosis (Ashish et al., 2020). Globally, one in ten women during their reproductive years (between puberty and menopause) are having endometriosis, which is about 176 million women population worldwide suffering from the disease (Hoyt & Falconi, 2015). This could be partially explained by the fact that the gold standard for diagnosis of endometriosis requires direct visualization of lesions at surgery followed by histological confirmation of endometrial glands and stroma in biopsies of suspected lesions (Parasar et al., 2017).

Additionally, other factors contributing to the diagnostic delay are treating pain with oral contraceptives or non-steroidal anti-inflammatory drugs and the assumption of dysmenorrhea as a regular event (Oladosu et al., 2018). Treatments are limited to hormonal therapy with many side-effects and complicated surgical removal of disease, which often needs to be repeated. Retrograde menstruation is a widely accepted theory; Dr. John Sampson explains the pathogenesis (Riazi et al., 2015b). This theory suggests that during menstrual blood and uterine tissue contractions, menstrual endometrial tissue flows back menstruating and implants through the fallopian tubes instead of out of the body in various sites, most commonly in the pelvis, into the oviducts and peritoneal cavity (Burney & Giudice, 2012). It was shown that 76–90% of all women experience this endometrial debris, including epithelial and stromal cells (Chen et al., 2013). However, only in endometriosis patients this menstrual tissue is able to adhere to peritoneal structures, developing a blood supply, and grows into an endometriosis lesion. Therefore, it is likely that the women who are developing endometriosis have genetic, biochemical or immune system dysfunction that does not allow the removal of the debris but rather facilitates menstrual

tissue adhesion to peritoneal structures and endometriotic lesion formation (Proctor & Farquhar, 2006; Sourial et al., 2014). However, new data suggests that endometriosis is linked to a disruption in the epigenetics regulators. Therefore, deciphering the changes mediated by epigenetic regulators in local chromatin structure during the stage-specific progression of endometriosis will highlight the role the epigenetics endometriosis progression (Guo, 2009). So, in this study, we have aim to first, identify the differentially expressed epigenes at different stages of endometriosis, secondly validation of these significantly altered genes in the patient's samples and correlating with disease progression, and then thirdly perform in vitro study to understand the functional role of some of these differently expressed epigenes in disease progression.

II. Materials and Methods

a) Classification Systems of Endometriosis

Numerous proposed systems to classify various forms of endometriosis exist mainly in the American Society of Reproductive Medicine (rASRM) (Lee et al., 2021), which is modified and renamed into Revised American Society for Reproductive Medicine classification of endometriosis (Adamson, 2011). All of these classifications divide endometriosis into four stages related to the increasing severity of the ovaries lesions, particularly the number of endometrial implants, their depth, and adhesions ; Stage I: 1-5 points indicates minimal disease, i.e., few superficial implants , Stage II: 6-15 points score indicates mild disease which includes more and deeper implants, Stage III: 16-40 points for moderate disease having many deep implants, small cysts on one or both ovaries and Stage IV: >40 points indicate severe condition with many deep implants, large cysts on one or both ovaries with dense adhesions (Johnson et al., 2017). Ethical clearance was obtained from our institution's institutional ethical committee before starting the study (No. Dean/2018/EC/936). Written informed consent from all the patients and healthy individuals were obtained.

III. Study Subjects

The present study was conducted between September 2017 to January 2021 at Department of Obstetrics and Gynecology, Sir Sunderlal Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi (25°20'N, 83°0'E). The data was selected from 18 fertile women as control, 34 infertile women with endometriosis. Data were randomly collected and the details of the lifestyle, habits, and familial history of patients were recorded. Patients with endometriosis were evaluated according to the revised American Society for Reproductive Medicine (rASRM) classification system (Rolla, 2019). All subjects were of Indian ethnicity from eastern Uttar Pradesh and Bihar, the two states of northern India. All the subjects were informed about the study and their consents were taken prior to the start of the study. Inclusion criteria include patients between 18- and 50-year age group, having their reproductive organs were included in the study. All patients of primary or secondary infertility who were subjected to diagnostic hysteron laparoscopy have endometriosis and BMI less than 32 kg/m2 were also included. Exclusion criteria includes patients with other causes of chronic pelvic pain, including infectious diseases, pelvic inflammatory disease (PID), adhesions due to previous surgeries or infections, who were excluded from this study.

IV. Clinical Characteristic of Subject Included:

Points included were the age, residence, physical and socioeconomic status of the subjects, type and duration of infertility, menstrual cycle-age of onset, frequency and its flow, an association of symptoms like dysmenorrhea, dyspareunia, chronic pelvic pain, urinary symptoms and their correlation to the stage of endometriosis. Physical examination of the study subject was done. Findings were analyzed concerning BMI, adnexal masses, mobility of uterus, and the presence of tenderness. TVS and MRI have been suggested for the detection of deep-infiltrating lesions. MRI and computed tomography, including ultrasound were evaluated for diagnosis. Diagnostic laparoscopy, a gold standard tool for direct visualization of the pelvis, helps in identifying the etiology of the patients' pain, was advised and evaluated. The laparoscopic staging was done based on the revised AFS scoring system (10), which categorizes the findings into four stages.

A. Tissue collection and RNA Extraction

Author Solid tissue samples were taken and immediately stabilized in RNAlater (Ambion™, RNAlater, Invitrogen, Germany) and then stored at −80°C. RNA extraction using the PureLink™ RNA Mini Kit. RNA were quantified using Nanodrop (Thermo Fisher Scientific) and stored at −80°C. Until qPCR. Reverse transcription and Quantitative real-time PCR (RT-qPCR) analysis of HOX transcript and other Gene levels. RNA samples were reverse-transcribed (RT) into complementary DNA (cDNA). cDNA was reverse transcribed from 1 ug of RNA by M-MLV Reverse Transcriptase (ABI) and was further diluted to 200 ng/µl. The diluted cDNA, 5 µl was used to quantify the transcripts in a 25 µl reaction volume with SyBr primers in a two-step quantitative real-time RTqPCR reaction performed using the CFX96 Optical Reaction Module C1000 Touch Thermal Cycler. The copy numbers were calculated using standard curves generated from genomic DNA and the relative gene expression levels were calculated using the comparative cycle threshold (CT) method by normalizing against the housekeeping genes; glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The calibrator was prepared with a mixture of cDNA from all samples, and consecutive dilutions were used to create a standard curve. Quantitative analysis of HOXA1_H _ex2, HOXA3_H_ex2, HOXA4_H,HOXA5_H_ex2, HOXA6_H_ex3, HOXA7_H, HOXA9_H, HOXA10_H, HOXA11_H, HOXB6_H, MLL2_H, DNMT1_H, DNMT3A_H,

DNMT3B_H, PRDM16_H_ex9, G9A_H, VENTX_H_ex3, GAPDH_H (Supplementary Table 1) was performed using a CFX96 Optical Reaction Module C1000 Touch Thermal Cycler and SYBR Green as the detection dye. HOXA10, HOXA11, HOXB6, MLL2, DNMT1 DNMT3A, and DNMT3B were quantified using the relative quantification method a calibrator. The PCR amplification efficiencies for the target and reference cDNAs were determined by different standard curves created by consecutive dilutions of the cDNA template mixture. The HOXA1_H _ex2, HOXA3_H_ex2, HOXA4_H,HOXA5_H_ex2, HOXA6_H_ex3,HOXA7_H,HOXA9_H,HOXA10_H,HOXA11 _H, HOXB6_H, MLL2_H, DNMT1_H, DNMT3A_H, DNMT3B_H, PRDM16_H_ex9, G9A_H, VENTX_H_ex3, GAPDH_H mRNA levels were expressed as multiples of these cDNA concentrations in the calibrator.

Gene	Forward	Reverse
HOXA1_H _ex2	5′CCCTCGGACCATAGGATTACAA3′	5′GCCGCCGCAACTGTTG3′
HOXA3_H_ex2	5′CGACAGCTCGGCGATCTAC3′	5′CGGGTACGGCTGCTGATT3′
HOXA4_H	5′GGTGGTGTACCCCTGGATGA3′	5′GACTTGCTGCCGGGTATAGG3′
HOXA5_H_ex2	5′GGAGTTCCACTTCAACCGTTACC3′	5′CGGAGAGGCAAAGAGCATGT3′
HOXA6_H_ex3	5′TCCCCTCCCAATGAGTTCCT3′	5′ACTCCTGCCCGCTGTGGC3′
HOXA7_H	5′CTTCTCCAGTTCCAGCGTCT3′	5′AAGCCAGTTTCCGCATCT3′
HOXA9_H	5′CCACGCTTGACACTCACACT3′	5′GCTCTCATTCTCGGCATTGT3′
HOXA10_H	5′AGGTGGACGCTGCGGCTAATCTCTA-3′	5′-GCCCCTTCCGAGAGCAGCAAAG-3′
HOXA11_H	5′CGCTTCAGAACTCGTTGCTTTG3′	5′CGGAAGAACTGGCAGTCTTTACCT-3′.
HOXB6_H	5′CCCAATCTCGGAT ATACTAC-3′	5′-CTCGGGTGGGGGAGCCAGGA-3′
MLL2_H	5′GGGCTGACCGCTTCCTCGTGCTTTAC-3′;	5′GGAGAACAGTTGTGGGGAGATGGGTC-3′
DNMT1_H	5′-TACCTGGACGACCCTGACCTC-3	5′-CGTTGGCATCAAAGATGGACA-3′
DNMT3A_H	5′TATTGATGAGCGCACAAGAGAGC-3′	5′GGGTGTTCCAGGGTAACATTGAG-3′
DNMT3B_H	5′-GGCAAGTTCTCCGAGGTCTCTG-3′	5′-TGGTACATGGCTTTTCGATAGGA-3′
PRDM16_H_ex9	5′-TGCCGCACGCAGAGAGCA-3′	5′-GGGAGGAGGCAAAACGAACAT-3′
G9A_H	5′-GCATGCAGCCAGTAAAGA-3′	5′-CTGTCGTCCAAAGTCAGA-3′
VENTX_H_ex3	5′-GGCTGGCCAGGGAGATG-3′	5′-TGCGGCGATTCTGAAAC-3
GAPDH_H	5′-CGACCACTTTGTCAAGCTCA-3′	5′-AGGGGTCTACATGGCAACTG-3

Table 1: Forward and reverse primer sequences and product size of the gene.

IV. Statistical analysis

Statistical analysis was performed using the SPSS IBM Statistics software version 23.0. Distributions of data sets obtained in the study were checked for normality using Kolmogorov-Smirnoff test. Means were separated using Tukey's test when data were normally distributed and variances were homogeneous (Bartlett's test for equal variances). The data were presented as Mean±SD. One-way ANOVA test was performed to for the statistical significance of the difference in mean values of variables among several groups. Two-tailed p-values less than 0.05 were considered statistically significant and p-values less than 0.0001 were considered to be highly significant. All the results were expressed in Mean ± SD.

V. Results

Age may be a factor for the occurrence of endometriosis, but the present study has shown that most of the women of reproductive age (32±8.08) suffering from endometriosis had a mean BMI value of 21.75±2.32 (Table-2). Women living in urban areas are diagnosed earlier for the disease, while those living in rural areas suffer from the disease much severely but are diagnosed in their later stages, probably due to lack of health knowledge.

Variable(N=221)		**Stage 1 (N=19)**	**Stage 2 (N=80)**	**Stage 3 (N=75)**	**Stage 4 (N=47)**	**P-value**
		N (%)	**N (%)**	**N (%)**	**N (%)**	
Age(years)	≤32	9(47.4%)	37(46.3%)	35(46.7%)	19(40.4%)	0.903
	>32	10(52.6%)	43(53.8%)	40(53.3%)	28(59.6%)	
Mean± SD	32.10±8.08					
Residence	Urban	7(36.8%)	21(26.3%)	28(37.3%)	16(34.0%)	0.487
	Rural	12(63.2%)	59(73.8%)	47(62.7%)	31(66.0%)	
BMI (kg/m^2)	≤22	6(31.6%)	43(53.8%)	32(42.7%)	25(53.2%)	0.215
	>22	13(68.4%)	37(46.3%)	43(57.3%)	22(46.8%)	
Mean± SD	21.75±2.32					

Table 2: Association of Demographic profile, BMI, and Biochemical findings with Endometriosis staging (Values are Mean±SD; χ2-values significant at P<0.05).

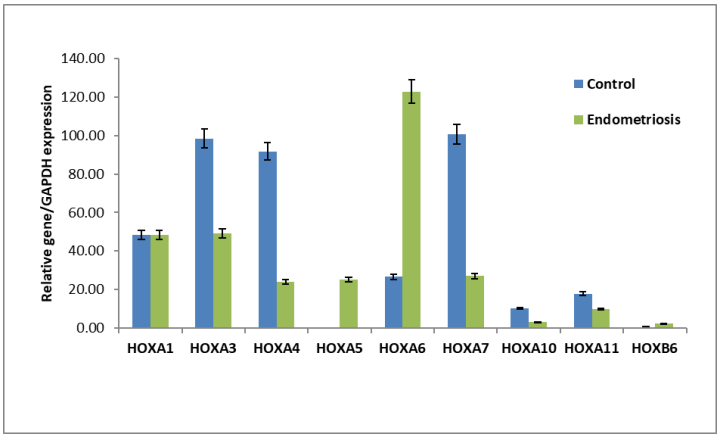

Figure 1: Relative Gene/GAPDH Expression in HOX10 Clusters.

The genes DNMT1, DNMT3A, DNMT3B, G9A, VNTX, PRDM, HOXA1, and HOXB6 were over-expressed in the ectopic endometrium as compared with normal control subjects or the eutopic endometrium of women with endometriosis, and their expression levels were correlated positively with each other.

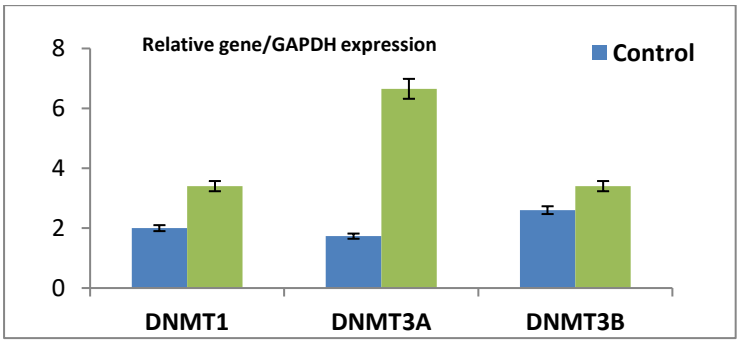

Figure 2: Relative Gene/GAPDH Expression in DNMT Family

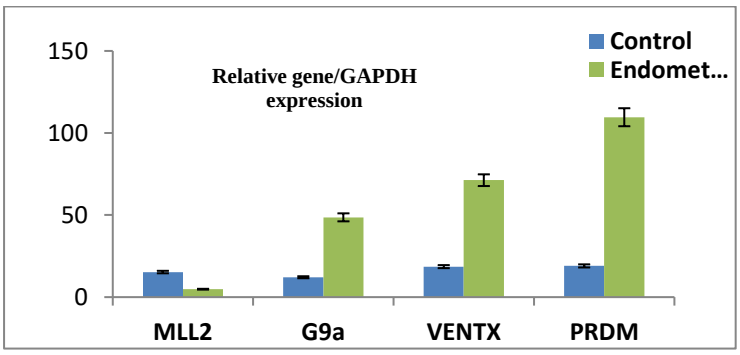

Figure 3: Relative Gene/GAPDH Expression in Different gene.

HOXA3, HOXA4, HOXA5, HOXA7, HOXA10, HOXA11, HOXB6 and MLL2 were downregulated ectopic endometrium as compared with normal control subjects or the eutopic endometrium of women with endometriosis, and their expression levels were correlated positively with each other.

V. CONCLUSION:

Over-expressed levels of DNMT1, DNMT3A, DNMT3B, G9A, VNTX, PRDM, HOXA1, and HOXB6 and Downregulation of HOXA3, HOXA4, HOXA5, HOXA7, HOXA10, HOXA11, HOXB6, and MLL2 in the ectopic endometrium and eutopic endometrium may play a role in patients with abnormal epigenetics which may lead to endometriosis.

VI. DISCUSSION:

HOX proteins are family of key transcription factors regulating embryogenesis in animals and are found to be linked with oncogenesis. The studied HOXA1 has been previously reported to be linked with breast cancers (A et al., 2016) Hox genes (HOXA1, HOXA2, HOXA11, HOXA13, HOXB1, HOXB13, HOXC13, HOXD4, HOXD10, and HOXD13) has been found to play diverse roles in Stem cell differentiation and function, and any dysregulation in these genes may cause cancer (Bhatlekar et al., 2018) It has been studied up to know that HOX genes are all together involved in proliferation, differentiation, migration and apoptosis processes and even it gets continued during carcinogenesis, having diversified roles even as transcription factors (Brotto et al., 2020) Here we find the differential expression of HOX genes (HOXA1, HOXA3, HOXA4, HOXA5, HOXA7, HOXA10, HOXA11, HOXB6), are duly altered in respect to control tissue expression profile, and these points together indicate that the HOX genes may be one of the causative factors in proliferation and inflammation of endometrial tissues on its eutopic regions during the diseases.

Further, DNA methylation, the one of the most studied epigenetic modification, that can be stably inherited through multiple cellular divisions, is induced by a family of DNA methyltransferases. In animal models, it has been studied in recent years that there is a relation between non-mutagenic environmental factors and epigenetic alterations subsequently leading to disease pathology and in these DNMT1 is most frequent studied methyltransferase (Kristensen et al., 2009) A study by Chai et al, 2019, has decipher the intensive inflammatory role of DNMT1, DNMT3a and DNMT 3b in LPS-induced human dental pulp cells (L et al., 2020) and DNMT1, was also found to has a correlation with decreased PPAR-γ and increased proinflammatory cytokine regulating chronic inflammation and atherosclerosis(L et al., 2020) Recently it was demonstrated that DNMT3a plays a critical role in modulation of mast cell responses, in acute and severe stimulations during a disease(Leoni et al., 2017) and the inflammatory response can be controlled via Rbm10 by mRNA splicing of DNMT3b gene(T et al., 2017) .The methyltransferase G9a promotes breast cancer recurrence, repressing pro-inflammatory genes in tumor's via elevated RIPK3 expression (Mabe et al., 2020) . The VENTX gene is altogether involved in myeloid cell differentiation and is highly expressive during myeloid leukemia (Rawat et al., 2010).

similarly, the MLL 2 gene is found to be most frequently mutated gene in many human cancers, while PRDM (PR/SET domain family) proteins are involved in cancer onset, invasion process and metastasis (De Mel et al., 2019).

The epigenetic modifications are complex, alters inflammatory responses and in future can be targeted to control inflammations and tissue damages, a pre-disposing effect of any disease. The study of different genes involved in epigenetic modifications will help us to understand pathophysiology of diseases, molecular molecules may be involved in the pathways, disease severity and relative phenological changes observed in new emerging diseases, leading to a new approach of disease diagnostic biomarkers. Thus, the differential expression of HOX genes in disease tissue in respect to diseased free tissue will help us to understand their putative roles during endometriosis disease progression. Further via cumulative result analysis one can derive the hypothesis that the dysregulation of HOX genes with DNMTs may leads to abnormal Cell self-renewal and differentiation processes in endometrium tissues.

VII. CONFLICT OF INTEREST

The authors have declared that the research was conducted in the absence of any commercial or financial relationships without any conflict of interest.

VIII. AUTHOR CONTRIBUTIONS

R.S. S.R., C.P.C and A. A have equally participated in the protocol development, collection and analysis of the data, manuscript writing, and final approval. A.S., K,. have participated in the study design, data evaluation, drafting, and analysis. S.M have contributed to data collection, manuscript concept, and design statistical data analysis.

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